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BREATHE (IN THE AIR):

PULMONARY IMMUNOLOGY IN MULTIPLE SCLEROSIS

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Cover: “Dark side of the lung” by Michael Hagemann-Jensen, inspired by the album cover “Dark side of the moon” by Pink Floyd.

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Breathe (In the Air): Pulmonary Immunology in Multiple Sclerosis

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ACADEMIC DISSERTATION

The public defence will be held on the 23rd of March at 9.00 am in the CMB Lecture Hall, Berzelius Vägen 21, Karolinska Institutet, Stockholm, Sweden

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To my family and loved ones

Breathe, breathe in the air
Don't be afraid to care
Leave but don't leave me
Look around and choose your own ground
For long you live and high you fly
And smiles you'll give and tears you'll cry
And all you touch and all you see
Is all your life will ever be
Run, rabbit, run
Dig that hole, forget the sun
And when at last the work is done
Don't sit down, it's time to dig another one
For long you live and high you fly
But only if you ride the tide
And balanced on the biggest wave
You race toward an early grave

-Pink Floyd

ABSTRACT

Multiple Sclerosis (MS) is a demyelinating disease of the central nervous system, with etiology still unknown. MS is thought to arise from a complex interplay between genetic and environmental factors. One of the most well established environmental risk factor is smoking, which confers a striking increase in risk of developing MS and especially in interaction with the risk allele HLA-DRB1*15 and absence of the protective allele HLA-A*02.

The major part of this thesis is focused on investigating the involvement of the pulmonary immune system in MS, and further to uncover underlying smoking associated changes that could elucidate on the role of smoking as a risk factor in MS. To characterize the lung immune cells, bronchoalveolar lavage (BAL) cells were obtained by bronchoscopy, from healthy volunteers and MS-patients, smokers and non-smokers. In **project I** we provide an initial characterization of our study cohort. We could observe that smokers carrying the MS specific risk allele HLA-DRB1*15 did not show a smoking-associated increase in macrophages defined in non-carriers. Smokers showed higher frequency of proliferating T-cells, while non-smoking MS-patients had increased levels of preformed CD40L in CD4+ T-cells. We could further provide a more in-depth characterization of pulmonary T-cells in MS-patients and smokers, in **Project III**. The majority of CD4+T-cells in both healthy and MS patients showed a tissue resident memory phenotype, characterized by expression of CD69 and CD44, while also expressing both CXCR3 and CCR6. Cells from healthy smokers showed an increased proliferative capacity and we also observed a significantly higher frequency of regulatory T-cells in the lungs of both healthy smokers and MS-patients compared to healthy non-smokers. When investigating the migratory profile of lung T-cells based on integrins VLA-4 and LFA-1, both implicated in MS pathogenesis, we found no upregulation of these in MS patients compared to healthy.

In recent years, it has been suggested that dysbiosis of the commensal microbiome in the gut is involved in the pathogenies of MS. The lungs also host a unique commensal microbiota, which recently was shown to be dysregulated in the autoimmune disease Rheumatoid Arthritis and pulmonary Sarcoidosis. In **Project IV** we investigated if the microbiota in the lungs of MS patients also show dysbiosis. We found that the microbial composition in the lungs of MS patients differed considerably compared to healthy controls, with increased richness and diversity. We could further report that MS patients also had altered expression and presence of the antimicrobial peptide human beta defensin-1 (hBD1) in the lungs.

In **Project II** we developed a novel method, called Small-seq, to study small RNAs, such as microRNAs (miRNA) from a scarce source of starting material; a single cell. Previously methods required large quantities of sample material in order to investigate small RNAs, which often can be a limitation to obtain in clinical samples, as well as average out biological variability and heterogeneity within populations. With Small-seq we are were able to capture different types of small RNAs from single cells, such as miRNA, snoRNA and tsRNA. Captured miRNAs revealed cellular heterogeneity in primed hESC, as well as being able to cluster and separate different cell types. The method implemented a masking strategy to efficiently limit capture of the highly abundant 5.8S rRNA, and incorporation of a unique molecular identifier allowed for molecular quantification of the detected small RNAs.

The work provided in this thesis concludes that the pulmonary immune milieu is altered in MS patients, thereby presenting the lungs as an organ of interest for further investigation into the pathology and potential therapeutic opportunities in MS. The described changes in immune cell composition between smokers carrying the MS risk allele HLA-DRB1*15 and non-carriers, could further shed light upon the mechanisms behind the impact of smoking as a risk factor for disease and in exacerbating MS. Herein we further provide the development of a novel technique to capture and investigate small RNA, such as miRNAs in single cells

LIST OF SCIENTIFIC PAPERS

* indicates equal contribution

I. Öckinger J*, **Hagemann-Jensen M***, Kullberg S, Engvall B, Eklund A, Grunewald J, Piehl F, Olsson T, Wahlström J.
T-cell activation and HLA-regulated response to smoking in the deep airways of patients with multiple sclerosis.
Clinical Immunology, 2016, 169, 114-120

II. Faridani OR*, Abdullayev I*, **Hagemann-Jensen M**, Schell JP, Lanner F, Sandberg R.
Single-cell sequencing of the small-RNA transcriptome.
Nature Biotechnology, 2016, 34(12), 1264-1266

III. **Hagemann-Jensen M**, Kullberg S, Müller M, Eklund A, Grunewald J, Piehl F, Olsson T, Wahlström J, Öckinger J.
Properties of resident T-cells in human lungs of smokers and multiple sclerosis patients.
(Manuscript)

IV. **Hagemann-Jensen M**, Hamza Bokhari M, Kruisbergen N, Piehl F, Olsson T, Grunewald J, Wahlström J, Segal LN, Scher JU, Öckinger J
Altered lung microbiota in Multiple sclerosis patients associated with local changes in immune environment.
(Manucript)

LIST OF SCIENTIFIC PAPERS NOT INCLUDED IN THIS THESIS

* indicates equal contribution

I. Small-seq for single cell small RNA sequencing.
Hagemann-Jensen M, Abdullayev I, Sandberg R, Faridani OR.
Nature Protocols (submitted)

II. Increased expression of inflammatory mediators in monocytes from smokers, after stimulation with cell wall polysaccharide from Streptococcus pneumoniae and other Toll Like Receptor ligands.
Öckinger J, Sundberg-Kövamees M, **Hagemann-Jensen M**, Kruisbergen N, Hamza Bokhari M, Grunewald J, Wahlström J.
Scandinavian Journal of Immunology (submitted)

III. Expression of MATE1, P-gp, OCTN1 and OCTN2, in epithelial and immune cells in the lung of COPD and healthy individuals.
Tove Berg, Tove Hegelund-Myrbäck*, Johan Öckinger, Xiao-Hong Zhou, Marie Brännström, **Michael Hagemann-Jensen**, Viktoria Werkström, Janeric Seidegård, Johan Grunewald, Magnus Nord, Lena Gustavsson
Respiratory Research (submitted)

IV. Common variants of T-cells contribute differently to phenotypic variation in sarcoidosis.
Rivera NV, **Hagemann-Jensen M**, Ferreira MAR, Kullberg S, Eklund A, Martin NG, Padyukov L, Grunewald J.
Scientific Reports. 2017 Jul 17;7(1):5623. doi: 10.1038/s41598-017-05754-7.

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PLoS One. 2016 Aug 30;11(8):e0161669. doi:10.1371/journal.pone.0161669.

VI. The selenium metabolite methylselenol regulates the expression of ligands that trigger immune activation through the lymphocyte receptor NKG2D.
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VII. The NKG2D ligand ULBP2 is specifically regulated through an invariant chain-dependent endosomal pathway.
Uhlenbrock F*, **Hagemann-Jensen M***, Kehlet S, Andresen L, Pastorekova S, Skov S.
Journal of Immunology. 2014 Aug 15;193(4):1654-65.doi: 10.4049/jimmunol.1303275

CONTENTS

LIST OF ABBREVIATIONS

AM	Alveolar Macrophage
AMPs	Antimicrobial Peptides
APC	Antigen-presenting cell
ASV	Amplicon sequence variant
BAL	Broncho alveolar Lavage
BBB	Blood Brain Barrier
CD	Cluster of Differentiation
CLR	C-type lectin receptors
CNS	Central Nervous System
COPD	Chronic Obstructive Pulmonary Disease
DADA2	Divisive Amplicon Denoising Algorithm 2
EAE	Experimental Autoimmune Encephalomyelitis
FOXP3	Forkhead box P3
GATA3	GATA Binding Protein 3
hBD	Human beta defensin
hESC	Human Embryonic Stem Cell
HLA	Human Leukocyte antigen
IFN	Interferon
IL	Interleukin
ILC	Innate lymphoid cell
MHC	Major Histocompatibility Complex
miRNA	MicroRNA
MS	Multiple Sclerosis
NLR	NOD-like receptor
OTU	Operational Taxonomic Unit
PAMP	Pathogen associated molecular patterns
pCD40L	Preformed CD40 ligand

PMT	Photomultiplier tubes
PRR	Pattern recognition receptor
qPCR	Quantitative Polymerase chain reaction
RA	Rheumatoid Arthritis
RLR	RIG-I-like receptor
RNA-seq	RNA sequencing
rRNA	Ribosomal RNA
RORγt	Retineic-acid-receptor-related orphan nuclear receptor gamma
S1PR1	Sphingosine 1-phosphate receptor-1
sdRNA	SnoRNA derived RNAs
snRNA	Small nuclear RNAs
snoRNA	Small nucleolar RNAs
Tbet	T box expressed in T cells
T_{CM}	Central memory T-cell
TCR	T-cell Receptor
T_{EM}	Effector memory T-cell
TGF	Transforming growth factor
Th	T helper
TLR	Toll Like Receptor
TNF	Tumor Necrosis Factor
Treg	Regulatory T-cell
T_{RM}	Resident memory T-cell
tRNA	Transfer RNA
tsRNA	Transfer RNA derived small RNAs
UMI	Unique molecular identifier

1 INTRODUCTION

1.1 PULMONARY IMMUNOLOGY

The human respiratory tract is an intricately organized system comprised of trachea connected to branched airways and bronchioles terminating in millions of alveoli, responsible for the primary function of the lungs, oxygen and carbon dioxide exchange. As a result of this highly branched unit, the lungs encompass the largest epithelial surface in the body that is exposed to pathogens, innocuous particles and toxins inhaled with each breath. To combat such pathogens the lungs have evolved a multi-layered host-defense and immune system, preventing entry into the body, while restraining inflammation mediated damage to ensure preserved gas exchange. These host defense barriers include layers of mucus, an abundance of fluid and antimicrobial molecules, and tight junctions between epithelial cells. Furthermore, crosstalk between resident alveolar macrophages (AMs), T-cells, dendritic cells and respiratory epithelial cells orchestrate restrained and well-controlled immunity[1].

1.2 MACROPHAGES

Macrophages constitute of a versatile and diverse set of cells that can be found in mammalian tissues, but each tissue residing macrophage population is distinct reflecting the different microenvironments, and their specific requirements. Macrophages are key players in tissue homeostasis through their most recognizable trait of phagocytosis, ingesting apoptotic cells and cell debris, together with eliciting tissue repair and control immune activation. As part of the innate immune response, they also serve as sentinels scavenging for microorganisms and protecting against infection and foreign particles and are capable of eliciting multiple inflammatory processes, depending on the type of stimuli. They also represent a link to the adaptive immune system by interplay with T-cells via antigen presentation, expression of co-stimulatory molecules, and secretion of cytokines and chemokines, thus enabling them to modulate the responses and initiate recruitment of immune cells to the site of inflammation.

It was initially believed that tissue resident macrophages originated solely from infiltrating bone marrow derived monocytes under steady state conditions, but lately that view has been challenged. Recent studies in mice have found macrophages to originate from embryonic progenitor cells that inhabit the early tissues and persist through the ability to proliferate and self-renew[2]. However, during inflammation, monocytes will be recruited by the residing macrophages to increase the immune defense and become what is termed monocyte-derived macrophages [3].

Macrophages have the ability to recognize and sense exogenous and endogenous danger via various pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), C-type lectin receptors (CLRs), NOD-like receptors (NLRs), and RIG-I-like receptors (RLRs). These receptors are able to recognize and react to a wide array of pathogen associated molecular patterns (PAMPs) on pathogens as well as danger associated molecular patterns (DAMPs) produced during cellular stress and/or tissue injury[4] . The response pattern differs for each of the PRRs. However apart from certain NLRs, activation of PRRs cause downstream activation of transcription factors that induce inflammatory responses. This culminates in the production of pro-inflammatory cytokines and type I IFNs, along with chemokines for cellular recruitment and antimicrobial peptides[5].

Activation of macrophages and subsequent polarization of these cells have typically been classified as either "classical" M1 or "alternative" M2; M1 being the inflammation driving macrophages, while M2 represents the opposite of more immune regulating and tissue repairing macrophages. However the M1/M2 theory does not translate very well across species and a paradigm shift towards a more spectrum based model is currently getting more evident[6,7].

1.3 ALVEOLAR MACROPHAGES

AMs constitute the main population of immune cells in the airway lumen of the lung and form the first line of defense against environmental challenges and pollutants. This tissue compartment is a unique microenvironment with a considerable influence on the tailored characteristics and turnover of alveolar macrophages, as adaptation is required to accommodate the ever-changing needs and challenges of the tissue. Similar to other tissue resident macrophages, AMs are long-lived and rely very little on circulating monocytes to self-maintain due to their ability to locally self-renew[8]. AMs have been described as being masters of contradictory functions, capable of distinguishing between different situations those requiring tolerogenic responses and those that require the initiation of an inflammatory response. They are responsible for the homeostatic functions of clearing and discarding cellular debris and inhaled particles, as well as regulating the levels of surfactant released by the epithelium. AMs are able to mount an inflammatory response but are most commonly in a hyporesponsive state, responding poorly to stimuli such as TLR ligands. Activation of AMs is tightly controlled and shaped by the microenvironment, via interactions with the commensal microorganisms and cell-cell contact with the airway epithelium, in order to limit any unwanted inflammatory responses[9]. Initiation of inflammation requires an override of the inhibitory mechanisms governing the AMs, which leads to a complex balancing act between activating and repressing signals[10].

1.4 T-LYMPHOCYTES

T-cells play a major role in the adaptive immune-system by protecting the host from pathogens and maintaining balanced host immunity. With their complex and highly diverse T-cell receptor (TCR) they recognize peptide antigens presented by cell surface proteins of the major histocompatibility complex (MHC) family, which include MHC class I and II. T-cells originate from bone marrow hematopoietic stems cells but develop and mature in the thymus. As they develop in the thymus the TCR is formed and the T-cells undergo positive and negative selection. These selection processes, respectively, ensure that the T-cells can recognize and interact with MHC, at an optimal strength, that no strong binding with presented "self-antigens" occurs, as well as directing the developing T-cell towards a $CD4^+$ or $CD8^+$ phenotype. T-cells that fail these selection criteria will be eliminated, with the exception of some T-cells that fail negative selection and are selected to become regulatory T-cells (Tregs). When the T-cells leave the thymus, they will have highly specific TCRs recognizing unique antigens presented by antigen presenting cells on either MHC class I or II molecules.

1.5 CD4+ T-CELLS

CD4+ T-cells recognize and respond to antigens presented by MHC class II on antigen presenting cells (APCs). If the needed co-stimulatory signals are present together with the cognate antigen, the CD4+ T-cell will become active, clonally expand and differentiate into a specific subset, depending on the required response needed, shaped by the specific cytokine signals present in the environment. These T helper (Th) subsets include Th1, Th2, Th9, Th17, T-follicular helper cells, as well as Tregs, which are capable of reciprocally regulating each other. Each subset can be defined by their distinct set of lineage-defining transcription factors and surface receptors that provide the ability to sense and respond to specific cytokines and chemokines. These lineage-defining transcription factors are induced in the naïve CD4+ T cells as a result of cytokine binding to specific receptors, initiating intracellular signaling through the JAK/STAT pathway. Differentiation of Th1 cells rely on interleukin 12 (IL-12) stimuli to upregulate expression of T box expressed in T-cells (Tbet), which is the key transcription factor involved in Th1 differentiation. Upon commitment via Tbet expression, Th1 cells will start to produce a specific signature of cytokines; interferon-γ (IFN-γ), tumor necrosis factor (TNF), and lymphotoxin-α. The activated th1 cells also express the chemokine receptor CXCR3, which then allows them to enter peripheral tissues. Th1 cells are pivotal in fighting intracellular pathogens and as a result of their IFN-γ secretion play an important role in macrophage activation. On the other hand, the primary role of Th2 cells is to fight extracellular parasites. Differentiation to the Th2 subset relies on the presence of IL-4 to drive their polarization by

initiating expression of the transcription factor GATA Binding Protein 3 (GATA3). This in turn induces the Th2 signature which is the production of cytokines IL-4, IL-5, and IL-13 cytokines, as well as expression of chemokine receptor CCR4 and prostaglandin D2 receptor CRTh2. At the same time, IL-4 induced signaling will inhibit the initiation of the Th1 and Th17 differentiation programs. Th17 are characterized by their production of IL-17 and have been shown to be important in fighting extracellular pathogens and fungi. Th17 cells are regulated by the transcription factor retinoic-acid-receptor-related orphan nuclear receptor gamma (RORγt), that is required for the production of IL-17 and drive the expression of CCR6 and IL-23R. The majority of all Th17 cells also express CD161 and CCR4. Transforming growth factor-β (TGF-β) and IL-6 are the two main cytokines needed to drive the Th17 specialization[11,12]. Both Th1 and Th17 subsets have been implicated in chronic inflammation and autoimmune diseases.

Tregs are primarily responsible for preventing any unwanted immune response, preserving tolerance to self and immune homeostasis. They are best identified by their "specific" transcription factor FOXP3, as well as expression of cell surface CD25 (alpha subunit of the IL-2 receptor). Subsequently markers such as CTLA-4, GITR and lack of the IL-7Rα chain, CD127, have also been proposed as Treg markers. Tregs are primarily generated in the thymus but can also be induced in the periphery. To date, it is still unclear how to distinguish thymus derived Tregs from those that are peripherally induced. Expression of the transcription factor Helios was initially believed to be a marker for thymus derived Tregs, but that has since been refuted, and Helios has instead been linked to various roles such as suppressive capacity and activation status of Tregs[13]. To exert their regulatory functions, Tregs can release an array of suppressive cytokines such as TGF-β, IL-10, and IL-35[12], as well as modulate and inhibit APCs via the inhibitory cell surface receptor CTLA-4[14]. Tregs are a vital part of maintaining a functional and balanced immune system and loss of Treg function or suppressive capabilities are indicated as contributing factors in many chronic inflammatory and autoimmune diseases[15,16].

1.6 CD4+ T-CELL PLASTICITY

Until recently, the consensus regarding T-cell differentiation was rooted in the concept that each specialized subset of T-cells was committed to a stable lineage phenotype. However, it seems that T-cell subsets retain the capacity to change their commitment and functional phenotype in response to changes in the polarizing environment, such as reactivation by altered cytokine milieu (Figure 1) or TCR engagement [17]. Th17 cells and Tregs are particularly able to adapt and change into more mixed or alternative phenotype. Both subsets have recently been

observed in functionally distinct pro- and anti-inflammatory roles, able to interchange phenotype and function [18]. Thus, under certain conditions, Tregs can acquire pro-inflammatory or effector T-cell functions at the expense of FOXP3 expression. These so-called exTregs have been shown to produce IL-17 or IFN-γ while losing their suppressive function[19]. With the advent of single cell technologies recent studies are uncovering a great deal of heterogeneity within seemingly homogenous populations of T-cell subsets. Th17 cells were shown to harness a wide array of heterogeneity ranging from pathogenic to regulatory functionalities[20,21]. In several inflammatory conditions and autoimmune diseases, such as Rhematoid Athritis (RA) and Multiple Sclerosis (MS), CD4+ T-cell plasticity has been identified by increased frequency of a pathogenic "hybrid" Th17/Th1 (hereafter referred to as Th17.1) subset able to co-produce IL-17A and IFN-γ, as well as high amounts of GM-CSF[22,23,24,25].

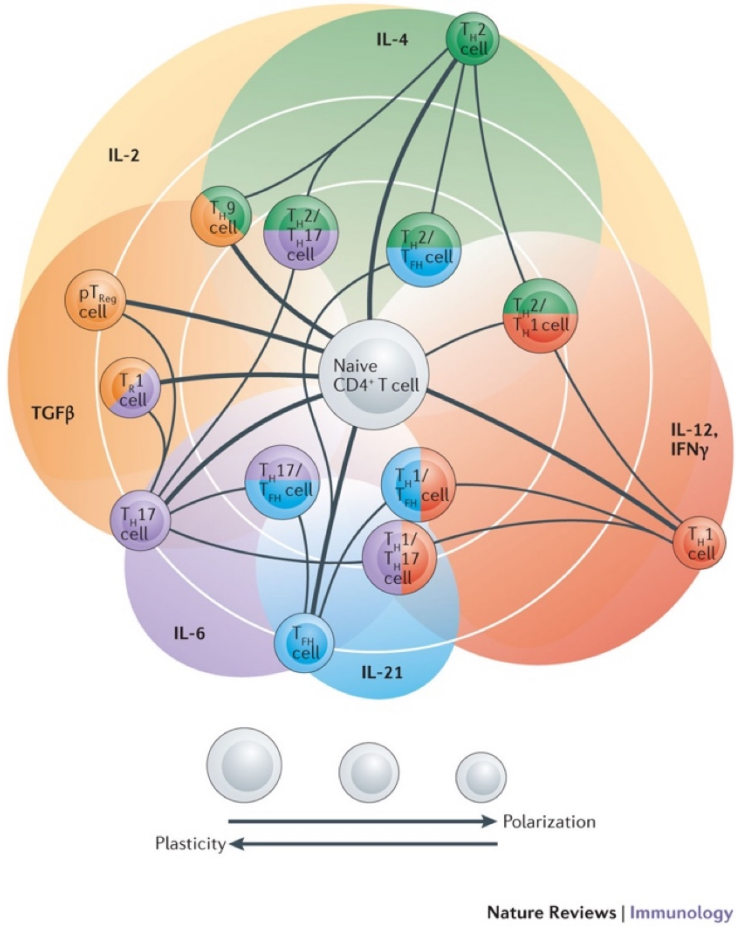

Figure 1: Simplified overview of key cytokines that promote polarization of naïve CD4+T-cells or plasticity between the subsets. The lines between subsets show known plasticity. (Dupage M & Bluestone JA, "Harnessing the plasticity of CD4+T-cells to treat immune-mediated disease" Nature Reviews Immunology 16, pages 149–163, 2016)[17]

1.7 CD8+ T-CELLS

CD8+ T-cells, also commonly referred to as cytotoxic T lymphocytes, are important for tumor surveillance and immune responses against intracellular pathogens, such as viruses and bacteria. In contrast to CD4+ T-cells, CD8+ T-cells recognize and respond to peptides presented by MHC class I molecules. When naïve CD8 T-cells get activated by recognizing presented antigen peptides, the antigen-specific CD8+ T-cells undergo vast clonal expansion and differentiates into effector T-cells. These effector CD8+ T-cells then recognize the antigen presented on infected or transformed cells, resulting in killing of these target cells via pathways involving either direct cell contact or cell killing mediated by release of cytokines TNF and INF-γ. Cytolytic pathways involving direct cell contact consist of the production and release of perforin and granzymes into the intercellular space. Perforin forms pores in the target cells membrane facilitating entry of granzymes, which cause cell death by engaging the apoptotic cascade. Apoptosis of the target cell can also be induced by Fas ligand expressed on the effector CD8+ T-cells interacting with Fas receptor on the target cell[26,27,28].

1.8 MEMORY AND TISSUE RESIDENT T-CELLS

After an infection or threat is properly eradicated the expanded effector T-cells undergo an apoptosis-induced contraction phase where most of the cells die off resulting in a small surviving subset of cells maturing into memory cells. These memory T-cells are long-lived, self-renewing and able to provide a rapid and enhanced response if the same antigen is encountered again. Classically these memory T cells have been subdivided into central memory T (T_{CM}) cells and effector memory T (T_{EM}) cells. T_{CM} cells express CD62L and CCR7 enabling them to access and reside in secondary lymphoid organs. In contrast, T_{EM} cells lack these receptors but instead express various tissue specific receptors enabling homing to peripheral tissue and provide immune surveillance. Both T_{CM} and T_{EM} recirculate and convey surveillance through blood, secondary lymphoid organs and peripheral tissue.

In the last decade it has become apparent that certain specialized lymphocyte populations remain fixed and persistently reside in peripheral tissues of the body. It still remains unclear whether a certain spectrum of tissue residency exists or to what extent and if the resident cells leave the tissue when encountering certain stimuli [29,30]. However, some migration within the constraints of the specific tissue and microenvironment has been observed to promote more efficient local immune surveillance[31,32]. Upon entry into the specific tissue the lymphocytes undergo a differentiation into resident cells based on the local signaling cues and microenvironment, which in mice can be distinguished by a set of transcription factors; Hobit and Blimp1[33]. Molecules associated with tissue egress are downregulated, while upregulation

and expression of CD69 is promoted to further establish tissue retention by suppression of sphingosine 1-phosphate receptor-1 (S1PR1) mediator of tissue egress[34].

The most well studied resident lymphocyte populations are tissue resident memory T (T_{RM}) cells, but other subsets of lymphocytes such as Tregs, non-conventional T-cells, and Innate Lymphoid Cells (ILCs) have also shown to be resident and phenotypically distinct from equivalent subset form in circulation. T_{RM} cells encompass both CD4+ and CD8+ T-cell populations that rely on different microenvironment cues, and appear to constitute most of the memory cells found in non-lymphoid tissues[35]. T_{RM} cells rapidly initiate and augment immune responses upon re-exposure to pathogens, by production of cytokines, such as IFN-y, and recruitment of circulating lymphocytes. It is still unclear how effective CD8+ T_{RM} cells are as direct killers and whether this is a primary mode of action[29]. Thus the primary role might be to quickly contain and delay the threat by profoundly modulating the local environment and trigger protective innate and adaptive immune responses, while recruiting for help[36,37].

As mentioned above, distinct residing Tregs are also present in different peripheral tissues. These tissue Tregs have been shown to harness specific phenotypes and functions beyond just serving as direct regulators of immunity, which impact the local tissue environment and homeostasis. One of the more studied phenotypes is the ability to influence and potentiate tissue repair in response to local cues and injury, for example in lungs[38] and muscle[39]. Furthermore, Tregs in visceral adipose tissue have been associated with insulin sensitivity and resistance, indicating functions for modulating metabolic homeostasis[40,41].

2 MICROBIOME AND THE IMMUNESYSTEM

We live in a symbiotic relationship with a large complex community of microorganisms that have coevolved and forged over millions of years. These microorganisms inhabit our bodily surfaces and carry out a vast variety of functions important for our physiology and protection. Nevertheless, it is critical to uphold compartmentalization and homeostasis of the microbiota to avoid potential pathologic and unnecessary immune activation. Physical barriers in the form of mucus layers and epithelial cells separate microbes and immune system, thus minimizing the direct exposure, while also producing various antimicrobial substances like antimicrobial peptides (AMPs). Despite this separation, the microbiota and immune system are in constant communication via a complex set of mechanisms in order to uphold homeostasis and optimal conditions for both parts[42,43].

The immune system is able to assert some "host" control over the microbiota composition through changes to the mucosal barriers, such as mucus production, pH and secretion of AMPs. Through microbial molecules and components, the microbiota can affect and interact with the immune-system, by interaction with TLRs present on epithelial cells and myeloid cells. These microbial molecules can also be sampled by DCs protruding into the lumen or penetrate the barrier causing a direct response[43]. Sensing microbiota especially through TLR signaling pathways are important for eliciting critical responses that maintains homeostasis e.g. repair of damaged intestinal epithelial[44].

2.1 LUNG MICROBIOME

The lungs consist of a vast surface area equipped with effective defense mechanisms to cope with the pressure and exposure from environmental microorganisms and particles. Similar to other bodily surfaces the lungs are also colonized by a variety of microbial communities. Compared to the gut, the lung has a relatively low microbial biomass or burden, possibly due to the difference in functional characteristics, a unique selection pressure, and reduced access to energy sources. The field of lung microbiome research is relatively young and thus many details pertaining to function and causality still remain unknown. It is currently unclear whether the lungs consist of stable residing populations of microorganism or are in a state of flux, constantly cleared and repopulated. This population or repopulation is hypothesized to happen through micro-aspirations from the upper respiratory tract, due to the observed similar microbial complexities[42,45–47].

Regardless, there still exists a delicate balance between host and microbe in lungs distinct from the upper respiratory tract[48,49]. The healthy lung of an adult appears to be primarily populated with the microbiota of the following genera; *Pseudomonas, Streptoccocus, Fusobacterium, Veillonella,* and *Prevotella*[50,51]. Changes in the lung microbiota composition have been linked to pulmonary inflammation and Th17 responses[49], inflammatory cytokines[52], and remodeled macrophage phenotypes[53]. Alterations in lung microbiome composition has also been observed in lung disease conditions such as asthma and COPD, with association to response to treatments and severity of disease, respectively[54,55].

2.2 ANTIMICROBIAL PEPTIDES

AMPs are an abundant array of short peptides, present in almost every living organism. In general, these peptides protect against invading microbes and pathogens in tissue. In humans, three main classes of functionally distinct AMPs exist, classified based on their amino acid composition and structure. These are the Defensins, Cathelicidins, and Histatins. Defensins can further be subdivided into alpha and beta Defensins. AMPs are expressed readily throughout the body, primarily by epithelial cells, neutrophils, macrophages, monocytes, and dendritic cells. The majority of AMPs are centered at the interface surfaces, like skin and mucosa, to maintain the homeostasis with the commensal microbiome as well as protecting against the constant exposure to environmental microorganisms[56].

The most common method of direct killing of pathogens is to employ their specific cationic charge, composition, and amphipathicity, to attach to the membrane bilayers and form transmembrane pores. The formed pores disrupt membrane integrity resulting in lysis of the targeted pathogen[57,58]. Apart from their antimicrobial activity, these cationic molecules have however also been demonstrated to be more complex and host a diverse range of immunomodulatory functions. AMPs have been shown to boast both pro- and anti-inflammatory capabilities, signal through chemokine receptors, and modulate TLR signaling. Both Defensins and Cathelicidins have demonstrated chemotactic capabilities both directly and indirectly via upregulation of chemokines and cytokines and can thus recruit leukocytes directly. Each type of AMP boasts a different chemotactic response that is selective towards the recruited cell type. Hence, to encompass their more immune mediated functions, they are also referred to as host defense peptides[59].

The lungs comprise of a vast surface area constantly exposed to potential pathogens. As such AMPs make an integral part of the first line of defense against pathogens, and dysregulation of AMPs is involved in pathogenesis and severity of several pulmonary diseases. For example, in

cystic fibrosis, COPD and asthma, differential regulation and function of AMPs were present in the lungs of patients compared to healthy individuals[60,61]. The causality and impact of these changes are still elusive and remains to be further explored. Additionally, dysregulation of AMPs has been observed in several types of diseases and inflammatory disorders. For example, in patients suffering from type 1 diabetes mellitus, serum levels of the only human Cathelicidin to date, LL-37, and Human beta defensin 1 (hBD1) are decreased compared to healthy individuals[62]. In patients with psoriasis, the levels of LL-37 and hBD2 were found to be raised, the latter correlating with IL-17A, an important driver of the skin pathology[62,63].

3 SMOKING AND INFLAMMATION

Cigarette smoking is a serious global health challenge affecting multiple organ systems and results in numerous smoking-associated diseases. The complex composition of cigarette smoke contains more than 4000 chemical substances, including nicotine, acrolein, carbon monoxide, phenols, toxic aldehydes oxidative compounds etc., which highly influences the respiratory system. The continual exposure to smoke induces various pathological effects and molecular changes in the lung epithelium and the immune cells connected to the lungs, which are associated with increased vulnerability to infections and risk of developing several disease, such as several cardiovascular diseases, COPD and asthma [64].

The influence asserted by smoking affects both the innate and adaptive immune system at a local and/or systemic level. Either induction or inhibition of the release of pro- and anti-inflammatory cytokines and mediators can be a consequence of smoke exposure[64]. A key mechanism behind the activation of immune cells by smoke exposure is activation of the NF-κB pathway, resulting in increased expression and secretion of TNF, IL-1, IL-6, IL-8, and GM-CSF. However, TLR induced NF-κB activation has also been observed to be inhibited by smoke exposure, resulting in a downregulation of the same cytokines[64]. The main instigators of the anti-inflammatory effects of smoking, nicotine, carbon monoxide, and phenols, demonstrate suppressive effects on inflammatory cytokines and mediators[65]. The effect of nicotine can be attributed to the interaction between nicotine and the α7 nicotinic acetylcholine receptor found on T-cells, B-cells and macrophages. These modulatory effects have an impact on different T-cell subsets and their responses, such as reduced activity of Th1 and Th17 cells caused by decreased expression of lineage specific transcription factors and reduced specific cytokine production[66]. Chronic exposure to smoke leads to an increased presence of macrophages in the airway lumen. Moreover, smoking can contribute to impaired function and changed morphology of alveolar macrophages, where cigarette smoke-derived particles remain trapped in the cytoplasm[67,68].

3.1 SMOKING AND AUTOIMMUNITY

More evidence is now being uncovered linking smoking to several autoimmune disorders as it has been identified as a risk factor and observed to play a role in RA, Systemic Sclerosis, MS, Crohn's disease and Systemic Lupus Erythematous[64]. In RA the interaction between the environmental influence of smoking and a specific genetic background increased the risk of the anti-citrullinated protein antibodies (ACPAs) form of RA by 21-fold[69]. Citrullinated peptides are autoantigens in RA and interestingly, increased expression of citrullinated proteins

and citrullinating enzymes have been observed in cells and fluid obtained by broncho alveolar lavage (BAL) from healthy smokers[70]. Contrary to the elevated level of autoantibodies related to smoking in RA, smoking has been shown to decrease the levels of anti-dsDNA titers in systemic lupus erythematosus suggesting a more immunosuppressive effect on auto-antibody creation[71].

4 MULTIPLE SCLEROSIS

MS is a chronic inflammatory disorder of the central nervous system (CNS) leading to demyelination, axonal degeneration and neuronal loss. The disease affects around 2,5 million people worldwide and is more prevalent in young adult women than men[72]. The disease is further sub-diagnosed into one of several common forms, relapse-remitting form (RRMS), secondary progressive MS (SPMS), and primary progressive MS (PPMS), based on clinical manifestations of the disease (Figure 2). Most frequently, MS patients will be diagnosed with RRMS, which is characterized by bouts of active disease, relapses, followed by periods of remission and clinical inactivity. However, most patients with this type of disease will eventually develop a more progressive form; SPMS. This disease state is defined by gradual worsening overlaid with or without relapses. A minority of MS patients will have the third form of MS, PPMS, which in contrast to the other types, has no periods of relapse-remission but proceeds as a continuous worsening from onset of disease. MS is a heterogeneous disease with great variation in clinical manifestations including physical disability, cognitive impairment and fatigue. These symptoms correlate with the diverse locations of the pathological inflammatory lesions in the CNS, arising from immune cell infiltration and subsequent CNS damage [72,73].

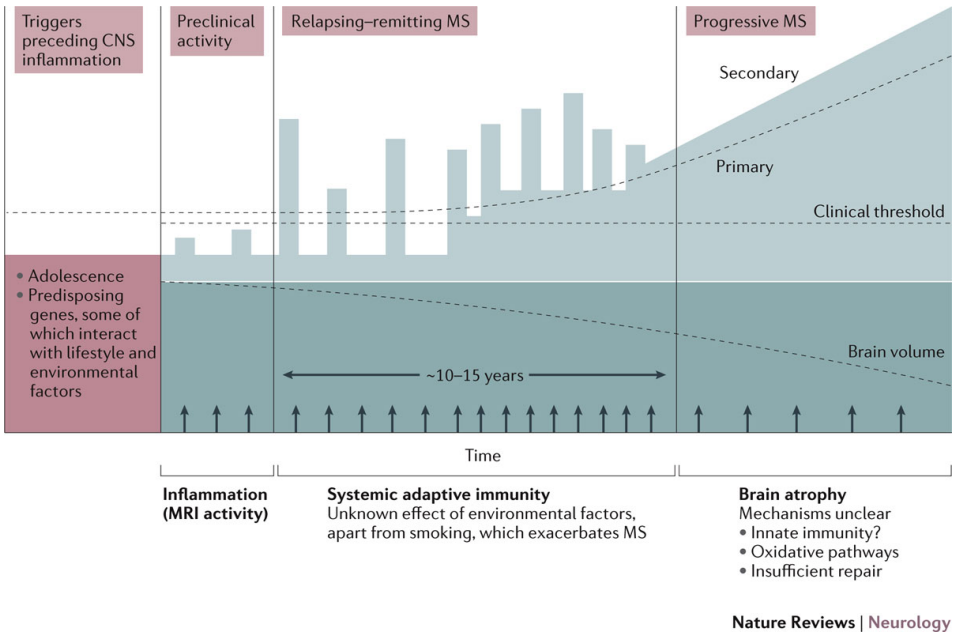

Figure 2: Overview of the disease course and the different progression forms of MS. (Olsson T, Barcellos LF and Alfredsson L, "Interactions between genetic, lifestyle and environmental risk factors for multiple sclerosis", Nature Reviews Neurology 13, pages 25–36, 2016)[74]

Diagnosing MS is based on the McDonald criteria. In brief, the diagnosis is established upon visualizing demyelinating episodes with magnetic resonance imaging (MRI) during two different time-points at different locations[75]. The first incidence of observed demyelination and suspicion of MS is classified as Clinically Isolated Syndrome (CIS). Furthermore, clinical symptoms are assessed by the Expanded Disability Status Scale (EDSS)[76], as well as screening of the cerebrospinal fluid (CSF) for the presence of and quantification of oligoclonal immunoglobulin G (IgG) bands (OCB)[77,78].

While etiology for developing MS is still unknown, it is regarded as an autoimmune disease, likely caused by a complex interplay between genetic and environmental factors. The precise mechanisms underlying disease pathogenesis are still unresolved. However, MS is believed to be initiated by defects in peripheral tolerance to an unknown CNS self-protein, causing the immune system to induce and perpetuate disease. Additionally, there exists a complex interplay between genetic and environmental factors that contributes to the development of MS. A clinical hallmark of MS is the manifestation of pathological inflammatory lesions, caused by the infiltration of immune cells from the periphery through the blood-brain barrier (BBB). T-cells in particular have been shown to play a central part in the pathogenesis of the disease. These auto-reactive T-cells activated by one or several auto-antigens in the periphery, have been shown to infiltrate the CNS and to be present at CNS lesions. The exact antigen(s) or trigger(s) of the autoreactive lymphocytes are still not known. However, several potential mechanisms exist. Those include loss of peripheral tolerance and a failure of regulatory mechanisms in the periphery, such as the inability of regulatory T-cells to suppress or discover unwanted auto-reactivity. Other potential mechanisms include molecular mimicry and bystander activation. As the disease progresses a state of chronic inflammation emerges and is likely maintained by local residing cells in the CNS, gradually replacing infiltration of peripheral immune cells[73].

4.1 INVOLVEMENT OF THE MICROBIOME IN MS

According to recent advances, the gut microbiome may contribute to pathogenesis and progression of MS. The gut microbiome profiles of RRMS patients demonstrate that they are distinctly different from that of healthy controls[79,80,81,82]. Germ-free mice lacking commensal bacteria were much less prone to develop experimental autoimmune encephalomyelitis (EAE), an animal model of MS, compared to mice colonize with microbiota, indicating a required role for the commensal bacteria for the induction of disease[83]. Additionally, dysbiosis of the gut microbiome was shown to cause a break in tolerance enabling encephalitogenic T-cells to trigger induction of EAE[84]. When human gut microbiota from MS patients was transplanted

into germ-free mice, disease severity of EAE was increased compared to mice transplanted with microbiota from healthy donors[85]. Moreover, healthy commensal microbiota were able to readily suppress EAE by decreasing pro-inflammatory Th1 and Th17 cells in favor of more regulatory and anti-inflammatory Tregs and suppressive macrophages[86]. Both human MS patients and EAE mice have an increased permeability of the intestinal mucosa, causing luminal contents to leak into the body[87,88]. Conversely, changes in gut microbiota in mice, could influence the BBB permeability due to lower expression of tight junction proteins[89]. This relationship between neuroinflammation and gut is also referred to as the gut-brain axis[90], and has gained increased interest in recent years.

4.2 PULMONARY INVOLVEMENT IN MS

The risk of developing MS is strongly linked to both genetic factors and environmental factors, with smoking being the strongest and one of the most established environmental risk factors for disease[74]. From the more than 200 identified risk loci associated with disease susceptibility, the human leukocyte antigen (HLA) complex exerts the strongest influence. HLA-DRB1*1501 confers the greatest risk for MS, odds ratio ~3, while HLA-A*02 is linked to a protective effect[91]. A striking interaction between gene and environment is observed when combining the prescence of HLA-DRB1*15, absence of HLA-A*02, and smoking, resulting in a 16-fold increased risk of developing MS [92]. Oral smokeless tobacco however has been shown to confer a protective effect on developing MS, indicating that lung irritation by smoking, rather than nicotine bears the responsibility for MS risk[93]. An increased risk of MS is also seen in individuals exposed to second-hand smoking[94], as well as in patients diagnosed with COPD [95], providing further evidence for a role of cigarette smoke exposure as a trigger for the development of MS. The association between second hand smoking and MS suggests that the link between MS and smoking could be due to non-specific lung irritation. In support of this, air pollution has been recently associated with increased relapse rates and inflammatory activity, although this hypothesis still needs further confirmation from replication and expanded studies[96,97].

Recently an EAE study using adoptive transfer in rats, implicated the lungs as a potential contributor to CNS infiltration of auto-reactive T-cells and induction of autoimmune disease. Briefly after adoptive transfer of the auto-reactive T-cells, these cells were observed homing to the lungs, where they would be reactivated or "primed", granting the ability to enter the CNS[98].

5 RNA TRANSCRIPTOMICS

5.1 QUANTIFICATION AND SEQUENCING OF RNA

RNA transcribed from the genome, is referred to as the transcriptome, and is essential for understanding cellular diversity, functionality, and molecular constituents in both health and disease. The transcriptome is composed of all transcribed species of RNA, including non-coding RNAs, and mRNAs. Quantifying RNA levels has long been used to identify and measure transcripts derived from active genes[99]. Earlier established methods of RNA quantitation rely on various ways to detect specific RNA abundance. For example, Northern blot utilizes hybridization with radioactive probes for detection[100] and quantitative real time polymerase chain reaction relies on the incorporation of fluorescent dyes in DNA from RNA converted to cDNA[101]. Similarly, microarrays use oligonucleotide probes to capture fluorescently labelled cDNA[102]. With the introduction of RNA sequencing (RNA-seq) and next generation sequencing came a method that could more precisely quantify RNA levels, at higher throughput, from lower starting material.

RNA-seq is a method to sequence fragments of cDNA converted from RNA. Due to the nature and chemistry of the most prominent commercial sequencers, long cDNA corresponding to long RNAs have to be turned into shorter fragments. The sequencing then produces millions of short reads, that represents partial read readouts of the original cDNA[103].

5.2 SINGLE CELL RNA-SEQUENCING

Due to technical constraints and limitations, conventional methods in detecting gene expression and transcriptome information were usually performed using “bulk” or tissue samples, containing thousands to millions of cells. Often however these populations of cells are not homogeneous. Recent advances in technology and the drive for higher throughput methods have made it possible to measure gene expression in hundreds and thousands of individual cells simultaneously. This allows for observation of cell-specific properties that might otherwise be masked in bulk population analysis due to being averaged out or diluted by all the cells in the sample. As such, single cell methods have become powerful instruments in dissecting the complexity of cellular composition in organ systems and identifying the specific cell-type transcriptomes. One major application of single cell RNA-seq is to identify rare cells and new cell-subtypes in an unbiased fashion from heterogeneous populations, that before would have been missed due to contaminating influence of the other cell types[104,105]. Further, this has enabled the possibility of discovering novel markers and gene signatures for specific cell-types. For example, rare cell-types in the intestine has been uncovered[106], as well as a more detailed

view of the early stages of embryonic development, cancer stem cells and neurodevelopment[107,108]. Another major advantage is the ability to observe fluctuations in the transcriptome and gain a better understanding of the dynamics involved in gene regulation and transcription, building our fundamental knowledge in RNA biology. Furthermore, transitions between cellular states (naïve vs primed stem cells) as well as tracing lineages across multiple cellular differentiation events are being uncovered[109]. This can lead to a novel understanding of regulatory mechanisms, intermediary states, and key genes/signaling pathways involved in various biological processes and disease states. Ultimately this fundamental knowledge may be applied to the development of better therapeutic targets or more efficient derivations/creation of stem cell populations for application in regenerative medicine.

A single cell, on average, contains 10pg of total RNA or lower, hence it is vital in single cell methods to minimize loss of RNA during processing as well as to maximize capture and conversion efficiency. To achieve this, cell lysis, cDNA conversion, and PCR amplification are carried out in the same tube, with addition of molecular crowding agents that effectively reduces the reaction volume, causing an increase in potential reaction rates and interations[110]. One of the most abundant species of RNA is ribosomal RNA (rRNA), which usually is not of interest. Hence to prevent this species of RNA from overwhelming both the cDNA library amplification and sequencing, bulk RNA usually undergoes a rRNA depletion step. However, this is only feasible when the amount of RNA is high as it results in general losses that therein are not practical in single cell methods. To avoid this, most single cell methods rely on only capturing the RNA containing poly-A tails, a feature not present in rRNAs. Furthermore, due to the low starting material, extensive amplification of the cDNA, usually by PCR, of the cDNA is necessary to create libraries prior to sequencing. This extensive amplification however comes at the cost of introducing PCR bias. A way to compensate for this bias is the implementation of a unique molecular identifier (UMI). UMIs are essentially a known stretch of random nucleotides, incorporated either before or at the cDNA conversion step and allow for counting and estimating absolute molecules[111,112].

This cutting-edge technology still faces some challenges which include: improving sensitivity and accuracy, reduce both biological and technical noise, as well as the development of new computational methods to complement the increased complexity of data generated[113]. Another important caveat, to date, is that the majority of single cell RNA-seq protocols are limited to the detection of RNA with poly (A) tails. A diverse substantial amount of RNA is not poly (A) tailed, and hence are not captured by most present single cell RNA-seq technologies[104,105]

5.3 SMALL RNAS

Small RNAs refers to a class of short (<300nt) non-coding RNAs, which are functional but not transcribed into protein. However, most of these small RNAs convey their biological function as a RNA-protein complex. It is now established that these small RNAs harness a remarkable variety of biological functions, including regulation of transcription and translation of multiple genes. Due to the regulatory nature of small RNAs, there is great interest for investigating and understanding the role of these molecules in terms of disease[114,115].

The most well characterized and studied small RNA is the species of microRNAs (miRNAs; ~22nt). Apart from miRNAs, a large variety of less characterized small RNAs have been described, including transfer RNA (tRNA), transfer RNA derived small RNAs (tsRNA; 30-34nt), small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs), snoRNA derived RNAs (sdRNAs) and more[114]. In MS miRNAs have been found to be dysregulated in a wide variety of body fluid, cells and tissues, including immune cells, neurons, brain tissues, and spinal cord. The dysregulated miRNAs seem to play an important role in the pathogenesis of MS and are proposed to serve as therapeutic targets and potential biomarkers for both disease diagnosis as well as treatments efficiency assessment[116–118].

5.4 SMALL RNA SINGLE CELL SEQUENCING.

Up until recently it was not possible to sequence small RNAs from single cells. However, Faridani et al. [119] (Project II) introduced, Small-seq the first method able to quantify and capture small RNAs at single cell level. This method utilized a ligation approach to capture several types of small RNAs including miRNA, tsRNAs and sdRNA, and incorporated UMIs for quantification. Further novel elements consisted of masking the most prevalent rRNA with a masking oligo, greatly reducing the amount of captured rRNA. This method was able to capture a vast array of small RNAs and was able to use expressed miRNA genes to readily cluster and separate different cell types[119].

6 AIMS

The overall aim of the work included in this thesis was to characterize the involvement of the pulmonary immune system in MS and identify smoking associated immune mediated mechanisms.

6.1 SPECIFIC AIMS

Project I: To investigate if the pulmonary immune system is altered in smokers or non-smokers diagnosed with MS.

Project II: To develop a method to quantify and investigate the role of small RNAs in individual cells.

Project III: To perform an in-depth characterization of residing lung T-cells in smokers and non-smokers and investigate changes in these subsets associated with MS.

Project IV: To investigate the lung microbiome in patients with MS and study compositional changes in relation to antimicrobial peptides and pulmonary immune components.

7 METHODOLOGY

This section will expand upon motivation and considerations in terms of certain methodologies used in the various projects. For more detailed description of the methodologies please see the respective papers.

7.1 STUDY COHORT (PROJECT I, III, IV)

Except for project II which focuses on sequencing the small RNA transcriptome of single cells, the studies included in this thesis relied on human material from MS-patients and healthy volunteers, smoking and non-smoking. All studies were approved by the Stockholm regional ethical board and all included subjects provided informed written consent.

To investigate the smoking associated immune events in the lungs of MS patients, both non-smokers and current smokers with normal lung function were recruited as well as current smokers and non-smokers with a MS diagnosis. Healthy volunteers were recruited by public advertising, while MS patients were recruited through the Neurology Clinic, Karolinska University Hospital, Stockholm, Sweden. All subjects underwent relevant clinical investigations, to ensure normal lung function and that no sign of airway infection or allergic symptoms were present at the time of bronchoscopy. Additionally, subjects were asked to fill out a questionnaire addressing general and pulmonary health, smoke and oral tobacco habits, and any current medication. All included MS patients fulfilled the McDonald criteria[120]. The MS cohort represents patients at various stages of the disease, disease course, and current treatments. All MS patients labelled as untreated in the various studies, are currently undergoing wash-out from previous medication, and should not be considered true untreated, but rather not on current medication. The majority of the included individuals were genotyped for HLA-DRB1 alleles from DNA extracted from whole blood samples, either using PCR-based methodology or estimated from SNP-genotype data. A minority of subjects were only genotyped for presence/absence of HLA-DRB1*15, a known risk-variant for MS.

Our healthy subjects are on average younger (median age 25 years) than our recruited MS patients (median age 39 years). There are two main reasons for this skewing of our study cohort. It is important to remember that both healthy individuals and MS patients are volunteering to undergo bronchoscopy and participate in this study. Neither have any direct clinical benefit from participation, and bronchoscopy is associated with a certain degree of discomfort. It is a general trend in studies involving human participants that a younger cohort is more likely to volunteer for study participation. However, MS is usually diagnosed between the age of 20-45, and thus naturally shifts towards an older population. Another reason for the age difference is

also bound in the interest of trying to discover immunological changes in young smokers that could foster an autoreactive environment, giving clues to how MS might develop and why smoking confers such a great risk factor. As such, age is always considered a confounding variable during data analysis.

7.2 SUBJECT SAMPLING (PROJECT I, III, IV)

The primary sampling procedure for the lung investigations was bronchoscopy. In brief, a flexible fiber optic bronchoscope is inserted nasally into the airways under local anesthesia. Mucosal tissue specimens are collected with forceps from the left lung compartment, either from the upper lobe, lingula or lower lobe and immediately flash-frozen. BAL is performed by instilling usually 5 aliquots of 50mL of sterile saline solution into the right lung. After each aliquot the fluid is gently aspirated and collected on wet ice until further processing. The BAL fluid contains both cells and soluble components. The majority of cells collected by BAL are alveolar macrophages (median 91.7% in healthy non-smokers) , but also includes lymphocytes (6%) and minor percentages of neutrophils, basophils, eosinophils and mast cells [121,122]. The cellular content of the BAL fluid adapts to the pulmonary health, and conditions such as bacterial infections, allergic asthma, and sarcoidosis, are characterized by marked increases in neutrophils, eosinophils and lymphocytes, respectively. Even though bronchoscopy is an invasive procedure it is considered very safe and can be performed on an outpatient basis, with only moderate sedation required[122]. Additionally, both blood and serum were sampled from each subject.

Project I, III and IV all rely on human samples, and especially primary cells retrieved by bronchoscopy. This brings about some challenges and limitations to the specific study designs. As bronchoscopy is an invasive procedure, recruitment of volunteers with very specific criteria or phenotype can be a challenge that requires time and dedication. This also makes it difficult or impractical to perform sampling at multiple time-points. The data presented in this thesis represents one time-point, a snap shot, of the specific state of health and disease and immunologic events. As such, caution is warranted when interpreting the results. A further complication is the fact that a BAL sample from a healthy individual on average yields 10-15 million cells that typically needs to be subdivided into various ongoing projects, hence the available material is often limited and constrained.

7.3 FLOW CYTOMETRY (PROJECT I, III)

Flow cytometry is a well-established method to characterize and capture various cellular components at a single cell level. The technique is based on laser-mediated detection of

fluorochrome conjugated antibodies, each exhibiting different excitation and emission spectra. Emission from the fluorochromes is captured by specific detectors, photomultiplier tubes (PMTs), set to a specific range of wavelengths. These PMTs convert the analog light signal into the digital signal, fluorescence intensity. When mixing multiple fluorochrome conjugated antibodies, their emission spectra are most likely to overlap, causing a specific PMT to detect emission from multiple fluorochromes instead of the designated one. To correct for this "spill-over" or spectral overlap between the PMTs, a compensation process is applied, subtracting an estimate of the emission from other flurochromes influencing the specific PMT. In addition to fluorescence emission, light scatter measuring cell size and complexity are captured by the forward and side scatter detectors.

7.4 QUANTITATIVE PCR (PROJECT III, IV)

For projects III and IV we used quantitative polymerase chain reaction (qPCR) to investigate the gene expression of select targets from RNA extracted from BAL fluid cells, mucosal tissue biopsies and stimulated alveolar macrophages. It has previously been reported that *PSMB2* and *RPL32* are most suitable housekeeping genes for expression studies in BAL, since they remain stable irrespective of age, gender, smoking, lung pathology and treatments[123]. However, we found that expression of *RPL32* varied significantly within our cohort, and in contrast both *PSMB2* and *HPRT1* showed stable expression. We thus used the average of *PSMB2* and *HPRT1* expression as stable value for reference gene expression. To calculate the relative gene expression or fold change the comparative Ct method was used[124].

7.5 TLR STIMULATIONS (PROJECT IV)

For project IV, we enriched BAL cells for AMs, and cultured these in the presence of known stimulants of TLR 1-6. Stimulation of TLRs elicits a complex signaling cascade involving the adaptor proteins MyD88, and TRIF. The latter is required for TLR3 signaling. Downstream signaling results in activation of the transcription factors NF-κB and AP1, which leads to the production of inflammatory cytokines. Apart from inflammatory responses, TLR activation can also influence several other biological processes including survival, antigen presentation and antimicrobial pathways[125,126].

7.6 DETECTION OF MOLECULES IN BAL FLUID (PROJECT III, IV)

Since the lavage is carried out in a large volume, the BAL fluid usually requires extensive up-concentration in order to detect cytokine and chemokines. The volume is measured after the up-concentration to determine and later adjust for the concentration factor. To facilitate analysis on multiple analytes simultaneously, we used bead capture assays based on the Luminex

technology. This is a sensitive method that relies on antibody coated beads of differing color to capture the targeted cytokines or chemokines. Specific biotinylated detection antibodies are then added to determine the fluorescence intensity or magnitude of signal. Quantification of the fluorescence intensity is done by plotting it on a standard curve derived from standard samples with known concentration.

To estimate the concentration of hDB-1 in the lungs (project IV) we utilized an enzyme-linked immunosorbent assay (ELISA), that much like the bead capture assay, captures the specific target molecule in a sandwich of antibodies and emit a magnitude of fluorescent signal based on the amount captured.

7.7 LUNG MICROBIOME SEQUENCING AND ANALYSIS (PROJECT IV)

DNA was extracted from the cell free BAL fluid, to perform amplicon sequencing on the highly conserved 16S rRNA gene hypervariable region 4. The 16S rRNA gene comprises of 9 hypervariable regions of different length, separated by conserved regions, making it possible to specific identify phylogenetic microorganisms. The V4 region is considered to be the most accurate and reliable region for classification and reproducibility[127–129]. Bacterial amplicons were produced using the established 515F-806R primer-pair, and sequenced at 150bp paired end, on an Illumina MiSeq[130]. As the V4 amplicon fragment is around 253bp, sufficient overlap between the paired reads occur to ensure higher quality data and reliable merging of reads.

To analyze amplicon sequence data we used the recently released algorithm Divisive Amplicon Denoising Algorithm 2 (DADA2)[131]. This method infers exact amplicon sequence variants (ASVs) and is able to distinguish biological sequence variants differing by one nucleotide. Prior methods relied on constructing Operational Taxonomic Units (OTUs) by arbitrarily clustering the sequencing reads differing less than a specific threshold; however, inferring ASVs by DADA2 have shown to provide higher reproducibility and better accuracy with fewer false positives[132]. The remaining computational analysis was based loosely on an adaptation of the published pipeline (Ben J Callahan et al. 2016)[133] and the R package Phyloseq[134].

7.8 SINGLE CELL RNA SEQUENCING (PRELIMINARY PROJECT)

To further investigate the cellular heterogeneity in pulmonary CD4+ T-cells, and the impact of smoking and MS, we single cell sort pulmonary CD4+T-cells, from healthy and MS-patients, smoker and non-smokers. Using index-sorting, phenotypic information based on surface markers is retained from each individual cell sorted into plates. This information might complement the transcriptional analysis and further assist downstream subset identification. Cells are stained, prior to sorting, with antibodies permitting us to identify the following

population based on surface phenotype; Tissue Resident (CD69+), Treg (CD25+CD127-), Th1 (CCR6-CXCR3+), Th17 (CCR6+CXCR3-) and Th1.17 (CCR6+CXCR3+), are sorted on a BD Influx FACS sorter. Synthetic RNA spike-ins (ERCCs) are added to each cell before being processed using the SMART-seq2 [135]. Spike-ins facilitate for downstream accounting of technical noise, and distinguish biological variation[136]. SMART-seq2 is able to capture full-length transcripts and is still one of the most sensitive single cell methods available. Obtaining full-length transcripts further allows for downstream computational reconstruction of the TCR and determination of clonality[137]. The Illumina compatible cDNA libraries are sequenced on an Illumina HiSeq 2500. The sequenced data is demultiplexed, mapped by STAR to the human genome (Hg38), and quantified using rpkmforgenes[138]. All downstream analysis is carried out using Seurat, an R package for single cell analysis.

8 RESULTS AND DISCUSSION

This section comprises of a summation of results and conclusions for each project. For more detailed discussions and results please refer to respective paper or manuscript.

8.1 PROJECT I

Smoking is a significant environmental risk factor for developing MS. This risk is further increased for individuals carrying the specific risk allele HLA-DRB1*15, while lacking the protective allele HLA-A*02. To investigate smoking induced immune mechanisms and alterations in human lungs associated with MS, and to potentially elucidate the underlying conferred risk of disease, we recruited healthy volunteers (smoking and non-smoking) and MS patients (smoking and non-smoking) to undergo bronchoscopy with BAL.

This project consists of the initial basic immune characterization of the majority of included individuals in our study cohort. This is also the first investigation into the pulmonary immune responses in MS patients, smokers and non-smokers. In this study we were able to identify smoking associated immune effects influencing the overall immune composition and proliferation. Smokers, both healthy and MS, had a radical increase in alveolar macrophages, which was specifically associated with HLA-DRB1 alleles. We could observe that smoking individuals carrying the MS risk allele HLA-DRB1*15 had significantly lower number of macrophages present, compared to smokers with other DRB1-alleles (Paper I, figure 4). Perhaps this difference in HLA-DBR1*15 individuals could be due to faulty recruitment in combination with smoking or shift in response to smoking. However, this finding suggests a direct link between smoke exposure associated immune changes and the major MS risk variant. Lung macrophages perform vital functions in removing cellular debris, and clearance of inhaled particle matter and irritants. This burden is greatly increased in smokers. It can be speculated that this decrease in macrophages could lead to a more severe perpetual inflammation due to inefficient clearance of particle matter and irritants.

We also discovered that in non-smoking MS patients, CD4+ T-cells harbored increased levels and expression of preformed intracellular CD40 ligand (pCD40L) compared to healthy non-smokers (Paper I, Figure 3). CD40L is an important co-stimulatory molecule for adaptive immunity, both for CD4+ T-cell stimulation and differentiation, as well as for macrophage activation and dendritic cell maturation[139]. Dysregulation of the CD40-CD40L pathway has been observed in several autoimmune and inflammatory diseases[140]. Additionally CD40L+ CD4+ T-cells are present at a higher frequency in blood of MS patients compared to healthy controls [141]. Further, increased levels of preformed CD40L have previously been shown in

effector and memory CD4+ T-cells as indicative of cellular activation and recent antigen exposure[142,143]. It can be speculated whether this increase in pCD40L in MS, is a sign of cells being in a more primed state, ready to produce a more rapid and powerful response.

Overall this work presents an introductory investigation into the immune environment in lungs of MS patients and smokers, providing evidence between smoking and disease associated changes in the pulmonary immune response. This study highlights the potential importance of the lung in the development of MS and establishes the need for a better understanding of smoking associated disease mechanisms in MS.

8.2 PROJECT II

The last years have proven single cell techniques very important and powerful for new insights to cellular heterogeneity, functions and identification of rare cell types. However, one obvious drawback has been that almost all the methods are limited to capturing and investigating only poly-A RNA; predominantly mRNA [144]. Small-RNAs refer to small non-coding RNAs that have emerged as important regulators of a wide range of biological post-transcriptional processes in both health and disease [115,145]. Investigating the function of small-RNAs has been previously limited to requiring large quantities of starting material. To further uncover cell-specific functions and regulation, there was a great necessity to develop a method sensitive enough to capture small-RNAs in single cells.

At the time of publication, we did not name the method, but we have since come to call it Small-seq. The Small-seq method for capturing small-RNAs in single cells and other sparse material is based on the ligation of adaptors to the 5' phosphate and 3' hydroxyl groups. To combat competition from rRNA, that also would get captured and ultimately occupy a lot of the reads downstream, we created a masking oligo towards the abundant 5.8S rRNA. By inclusion of this masking oligo we were able to greatly reduce the amount of captured rRNA (Figure 3).

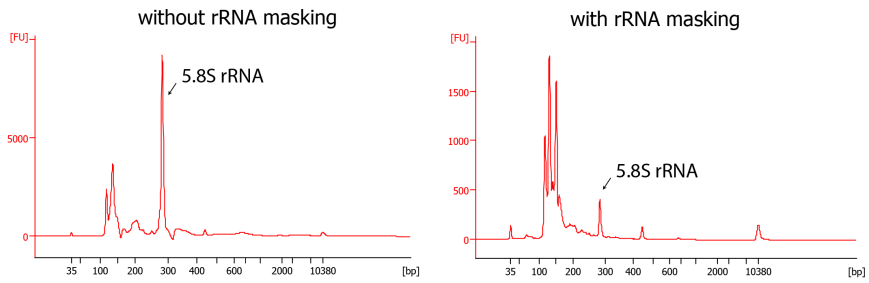

Figure 3: Bioanalyzer traces from small-RNA libraries of primed hESC cells created without (left) and with (right) rRNA masking of the 5.8S rRNA.

We incorporated an eight nucleotide long UMI, on the 5' adaptor, to be able to quantify the number of small RNA molecules captured, as well as to remove technical biases introduced by downstream PCR amplification. The method was originally developed without a gel size selection step which allows for automation of the method, however we have observed, after publication that a size selection step decreases the amount of contaminating adaptor dimers, thus greatly enhancing the quality of downstream sequencing output. To complement the Small-seq method and the data created, a computational pipeline was developed to support the analyses (https://github.com/eyay/smallseq).

To validate the method, we investigated the expression and composition of small-RNAs in naïve and primed human embryonic stem cells (hESC) as well as in HEK293T cells. We chose to focus on miRNA, tsRNA and sdRNA, as they were among the most abundant small RNAs (Paper II Supplementary Figure 4a). We were able to capture on average 3800 miRNA, 3500 tsRNA and 600sdRNA molecules per cell (Paper II, Figure 1b). When investigating the small RNAs of interest, we were able to show differential expression of miRNAs in single cells consistent with similar miRNA profiling in bulk samples of naïve and primed cells. We could also reveal cellular heterogeneity by variation in miRNA expression exclusively in primed hESC, when compared to naïve cells. In contrast, we did not observe many tsRNAs and sdRNAs differentially expressed between naïve and primed hESC. Furthermore, the miRNA profiles generated, revealed a great potential to robustly cluster and separate different states of pluripotency and cell types (Paper II, Figure 1k), comparable to that of mRNAs (Paper II, Figure 1l). To further evaluate the ability to separate cell types by their miRNA profile we included several types of glioblastoma cells (Figure 4).

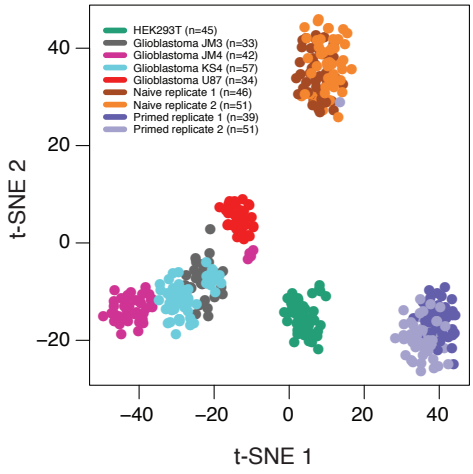

Figure 4: Clustering with t-distributed neighbor embedding(t-SNE) of naïve, primed, HEK293T and glioblastoma cells based on the captured mature miRNAs expressed at least

once in at least two cells. The number of cells for each type and replicate is indicated in the parenthesis in the legend (Paper II, supplementary figure 10)[119].

Two important features to establish in a new method are sensitivity and quantitative accuracy. Here the sensitivity was measured as the number of mature miRNAs detected. To evaluate the sensitivity, we compared the detected levels of mature miRNA, expressing more than 1 molecule in a single HEK293T cell, to serial dilutions of total HEK293T RNA ranging from 1000ng to 0,01ng. For the dilution range between 1000ng – 1ng the miRNAs detected were fairly constant; around 450 different mature miRNAs were expressed more than 1 molecule/cell. Below 1ng total RNA we detected technical losses. At 0,01ng, approximately the amount of RNA estimated to be in a single cell we detected 40% mature miRNAs, compared to the higher dilutions. This was comparable to the detected levels found in a single HEK293T cell, hence we could conclude that Small-seq has 40% sensitivity, capturing roughly 40% of mature miRNAs in a single cell.

In conclusion we developed a novel, sensitive method to analyze and quantify small RNAs from single cells and sparse material. Captured small RNAs, in particular miRNAs, were able to clearly discern cell-type and cell heterogeneity. By being able to investigate small RNA in single cells we will be able to learn more about their regulation and how these are affected by pathological conditions. This method is specifically designed to capture small RNAs, and as such information about long non-coding RNAs and mRNA is omitted. To fully explore the biology of the single cell transcriptome and its regulation, Small-seq could be combined or used in conjunction with other available single cell methods, to also obtain both mRNA data together with information about small RNAs.

8.3 PROJECT III

The work presented in Project III, is a continuation of Project I; providing a more detailed examination of the T-cell mediated immune response in smokers and MS patients. Interestingly we were able to identify that most T-cells in healthy lungs express both chemokine receptors CCR6 and CXCR3; which are associated with Th17 and Th1 phenotypes, respectively. This has previously been reported in lung diseases like asthma and sarcoidosis. We also observed smoking associated changes in the composition of residing T-cells, defined by CD69+. In particular CD69+ CD4+ T-cells expressing both CCR6 and CXCR3 chemokine receptors were significantly reduced in the lungs of smokers, in favor of CCR6+ and double negative CD4+ T-cells (Project III, figure 1E and Table 2). We could observe that the CD69+ T-cell population of cells overall showed higher proliferation than CD69- T cells, which was significantly

increased in the lungs of healthy smokers. Smoking also increased the percentage of proliferating CD69- CD4+ T-cells in both healthy and MS. (Project III, figure 2D-E).

It is difficult to conclude whether these compositional changes are due to shifts in the on-site proliferation or recruitment of T-cells. In asthmatic BAL CD4+ T-cells, both CCR6 and CXCR3 have been shown to be internalized after antigen exposure in vitro, and this could also be a possible explanation for increased composition of CCR6 and CXCR3 positive CD4 T-cells in smokers[146]. We analyzed expression and presence of ligands for CCR6; CCL20 and CXCR3; CXCL9,-10,-11, in BAL cells, mucosal tissue biopsies and BAL fluid. In all instances levels of the ligands CCL20, CXCL9,-10,-11 were decreased in smokers compared to non-smokers (Project III, figure 4).

As we previously had reported in Project I, CD4+ T-cells in BAL from MS patients had increased levels of intracellular pCD40L. In Project III, we discovered that pCD40L was more prevalent in CD69+CCR6+CXCR3+ expressing CD4+ T-cells, which still was significantly increased in MS patients (Project III, figure 3). A recent study has suggested that CCR6+CXCR3+ expressing CD4+ T-cells represents a population of persistent multifunctional cells important for controlling latent infections or chronic inflammation[147]. Increased pCD40L in these double expressing cells could be part of a functional repertoire to quickly react and control infection and inflammation.

Furthermore, we could discover that both healthy smokers and MS patients had increased occurrence of Tregs in BAL compared to healthy non-smokers (Project III, Figure 6). Tregs are important to maintain homeostasis, peripheral tolerance and control ongoing immunological responses. This increase could both indicate and serve as a response to ongoing underlying immunological mechanisms.

One of the few biological examples demonstrating the involvement of lungs in autoimmune neuroinflammation, was the lung specific “priming” or reprogramming of autoreactive T-cells observed by Odoardi et al. in their EAE rat model of the disease resulting in autoreactive T-cells with a distinct migratory profile[98]. To investigate if T-cells in MS patients had increased migratory potential, we tested for expression of known integrins, VLA-4 and LFA-1, responsible for the traversal of autoreactive T-cells across the BBB in humans. Somewhat surprisingly, we did not see any upregulation of these integrins in pulmonary T-cells from MS-patients compared to healthy subjects. In addition, smoking significantly decreased the expression of integrins. However, when comparing BAL T-cells with T-cells isolated from blood we saw significantly increased levels of the tested integrins in BAL, except for LFA-1

in MS patients. In mice, VCAM-1 (ligand of VLA-4), and ICAM-1 (ligand of LFA-1) were shown to be constitutively expressed in the lung compartment. VCAM-1 was predominantly expressed in the bronchioles, while ICAM-1 on extravascular cells. Both integrins and their ligands were responsible for controlling recruitment of immune cells in response to infection and subsequent interactions with the lung tissue and bronco alveolar compartment[148]. It can be speculated the autoreactive T-cells in the lungs acquire the function to traverse the BBB, due to mechanisms vital for normal lung function and response to infection.

This project presents a more thorough investigation of lung T-cells under physiological conditions as wells as in the context of smoke exposure and disease. We provide a detailed insight into the suggested multifunctional lung resident T-cells. Furthermore, we were able to uncover several smoking and disease associated changes to migratory and regulatory mechanisms in lungs. Identifying disease specific changes in lungs, might provide new insight about the etiology and progress of MS, and open up new avenues for therapeutic strategies, uncovering possible ways the lungs can be exploited in the treatment of MS patients.

8.4 PROJECT IV

Recent studies have attributed dysbiosis and alterations in the gut microbiome to MS and neuro-inflammation[79,149]. In EAE models, it has been shown that dysbiosis can cause a break in tolerance and induce disease, or increase disease severity [84,85]. Other organs, such as the lung, have been shown to harbor a microbial flora. The lung microbiome has recently been shown to have a reduced diversity in patients with Sarcoidosis and RA[150]. Hence, we were interested in studying if dysbiosis also was present in lungs of MS patients, and if any changes in microbiota composition were associated with any immunological characteristics.

Bacterial DNA extracted from BAL fluid was sequenced for the 16S rRNA hypervariable region 4. The healthy cohort included in this project has already been compared to Sarcoidosis and RA patients. As such, the samples were sequenced together with the sample from MS patients[150]. We could observe that MS patients had significantly higher species richness and evenness compared to healthy controls, regardless of smoking status (Project IV, Figure 1). This was in stark contrast to the diminished bacterial community observed in Sarcoidosis and RA. Nevertheless, when we investigated the relative bacterial composition of MS patients further, we found similar decreases in *Actinomyces, Chryseobacterium,* and *Prevotella* genera as reported in RA (Project IV Figure 3c) [150].

Interestingly we observed similarities between changes in the lung microbiome of MS patients and MS specific changes reported in the gut. Most strikingly, the genus *Acinetobacter* had a

higher prevalence in MS and was also one of the most important drivers of the differences in microbiota composition between healthy and MS. *Acinetobacter* has been implicated various aspects of MS disease, including exacerbating EAE symptoms and severity, inducing pro-inflammatory response in human T-cells, and encoding peptide sequences which are able to mimic myelin basic protein, and myelin oligodendrocyte glycoprotein[85,151]. Higher titers of anti-*Acinetobacter* antibodies have also been observed in MS patients[151]. Most notable we could also identify microbial changes that positively correlated with disease severity (*Streptococcus*) and disease duration (*Faecalibacterium*).

To complement the discovery of dysbiosis in MS patients we wanted to investigate whether any differences in the antimicrobial defense could also be found. We screened BAL cells and mucosal tissue biopsies for expression of AMPs. We found that *DEFB1* mRNA was significantly more expressed in BAL cells and the protein *hDB-1* present in significantly higher amounts in BAL fluid from MS patients compared to healthy subjects. This increase in *hDB-1* correlated with the changes of *Rhodanobacter* and *Corynebacterium*. Interestingly, *Corynebacterium* have been shown to regulate TLR3 response [152]. We could show in AMs that TLR3 stimulation by PolyI:C upregulated *DEFB1* mRNA expression, suggesting potential occuring regulation between *Corynebacterium*, TLR3-signalling and release of hDB-1.

A limitation of this study is the potential cross contamination during bronchoscopic sampling, which might act as a confounder in low biomass samples like lung microbiome samples[153]. No oral samples were obtained at the time of bronchoscopy nor were any bronchoscopic environmental controls obtained to verify possible cross contamination. However, contamination of throat or oropharyngeal microbiota has been shown to not influence sampling by bronchoscopy [154–157].

To conclude, we were able to identify a dysbiosis in MS lungs, with striking similarities to those previously reported in the gut of MS patients as well as in RA and Sarcoidosis. Furthermore, we report MS specific changes to AMP response that correlated with changes seen to the microbiome in the lungs of MS patients. We were also able to associate the observed microbiome changes in MS patients with clinical and disease specific characteristics.

8.5 PRELIMINARY DATA

To better understand the changes and findings from Project I, III and IV, especially with regard to residing T-cell populations and specific functionalities in the lungs, we decided to investigate the single cell transcriptomics of pulmonary CD4+ T-cells. Around 3000 CD4+ T-cells from

healthy individuals and MS patients have been single cell index sorted and sequenced (Figure 5).

Subjects	Healthy Individuals		MS Patients
	Never smokers	Smokers	Never-smokers
	H-NS	H-S	MS-NS
Individuals	2	2	2
#cells	576/480	480/384	480/480
Total cells	2880		

Index sorting	Gating (singlet & Live/dead exclusion
Th17	CD4+CCR6+CXCR3-
Th1.17	CD4+CCR6+CXCR3+
Th1	CD4+CCR6-CXCR3+
Th0	CD4+CCR6-CXCR3-
Treg	CD4+CD25+CD127-
Teff	CD4+CD25-CD127+
CD69	CD4+CD69+

Figure 5: Shows current distribution of sequenced cells as well as the markers used for index sorting and characterization.

Utilizing index sorting allows us to discriminate and classify each sorted cell by surface phenotype (Figure 6).

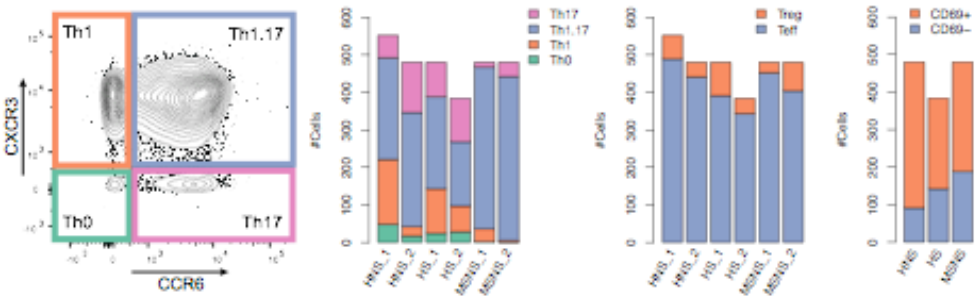

Figure 6: Distribution of sequenced cells characterized by surface marker analysis from index sorting.

More than 1500 single cells from another three individuals have already been sorted and await cDNA conversion and sequencing. From the very preliminary data gathered and quality filtered, we were able to cluster and identify multiple populations of CD4+ T-cells in healthy non-smokers based on their highly variable expressed genes (Figure 7).

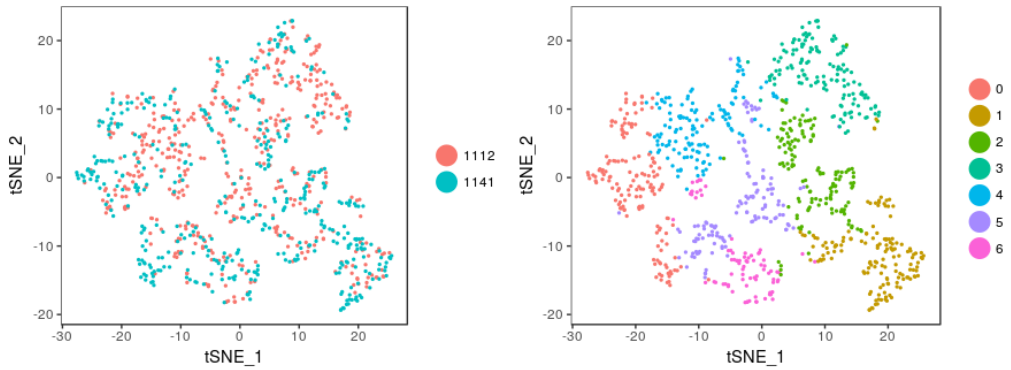

Figure 7: Clustering of CD4+ T-cells from two healthy non-smokers with t-SNE showing batch corrected overlap of the two individuals (left) and clusters identified based on expressed genes. (right).

The single cell data presented here is still in a very preliminary state. Both acquisition and analysis of the data is currently ongoing for the project. However, it does reflect a continuation of Project I and III as well as showcasing one of the future directions chosen, to further investigate and hopefully broaden our knowledge of the human pulmonary CD4+ T-cells and their heterogeneity. Furthermore, by utilizing single cell sequencing we hope to identify MS associated changes in CD4+ T-cells populations and functionality, as well as examine the clonality of such subsets.

9 CONCLUSION AND FUTURE PERSPECTIVES

The majority of the presented work in this thesis is part of an extensive and rigorous ongoing investigation into the involvement of the pulmonary immune system in MS. Furthermore, the studies presented are aimed to elucidate the effects of smoking in the development and/or progression MS. Project I, III and IV use various approaches to identify significant changes to the immune environment and host defense in the lungs of MS patients and young smokers. Overall, our findings conclude that the lungs are a peripheral organ of interest, which may aid in our understanding of the development and etiology of MS. This thesis also presents the development and work of a highly sensitive, novel method, to detect small RNAs in single cells with the potential to further extend our knowledge of small RNA biology and implications in disease.

In project I we observed less macrophages accumulation in the lungs of smokers carrying the MS risk allele HLA-DRB1*15. Further studies investigating this relationship are warranted since the interaction between smoking and carrying the HLA-DRB1*15 allele is one the most prominent increases in risk of developing MS. It would be interesting to further infer whether this gene-environment interaction causes changes in macrophage chemotaxis and functionality. Additionally, it would be worthwhile to determine the consequences of the lowered macrophage percentage in smokers, in terms of impact of host defense and ability to properly clear and dispose of smoke associated particles. The latter could potentially cause a more severe state of chronic irritation and inflammation.

Project I and III reported several changes in the composition and potential function of CD4+ T-cells in both MS and smokers. To further elucidate on composition and possible functions of CD4+ T-cells, not only in MS but also in young smokers and healthy individuals, we decided to use single cell transcriptomics to further examine lung CD4+ T-cells in health and disease.

When we investigated the difference in lung microbiome between healthy individuals and MS patients in project IV, we uncovered dysbiosis in MS patients with similar traits in lungs of RA, as well as in the MS specific changes in the gut. Despite establishing a relationship between local immune changes, and disease specific parameters, future experiments are needed to establish a causal role of both changes in microbiome composition as well as changes in AMP presence. In addition, *Acinetobacter,* which has been shown to produce mimicry peptides of myelin components was more abundant in MS lungs[151], suggesting a possible role of *Acinetobacter* in MS development. It could be important to further examine microbiotic

changes in MS patients, from a molecular mimicry perspective, and their potential to activate the local immune system.

Project II culminated in the development of Small-seq, a sensitive and novel method to study small RNAs in scarce material and single cells. Investigating and profiling small RNAs in single cells, complex tissues, and clinical biofluids, will help extend our knowledge about the biology of small RNAs and their regulatory roles in cells, as well as facilitate possible biomarker discovery. This could, for example, be implemented in pulmonary T-cells, analogous to the single cell mRNA sequencing presented in this thesis. Another application would be analysis of the miRNA transcriptome of immune cells from the CSF, a highly relevant clinical material in MS research. Combining Small-seq with expression of long RNAs/mRNA will further increase the insight into regulatory relationships between small RNAs and regulation of transcription in single cells.

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